INFECTION AND IMMUNITY, Jan. 2000, p. 6–12 Vol. 68, No. 1
0019-9567/00/$04.00+0

Characterization of PaxA and Its Operon: a Cohemolytic RTX Toxin Determinant from Pathogenic *Pasteurella aerogenes*

PETER KUHNERT,* BÉNÉDICTE HEYBERGER-MEYER, JACQUES NICOLET, AND JOACHIM FREY

Institute of Veterinary Bacteriology, University of Bern, CH-3012 Bern, Switzerland

Received 10 May 1999/Returned for modification 24 August 1999/Accepted 27 September 1999

Pasteurella aerogenes **is known as a commensal bacterium or as an opportunistic pathogen, as well as a primary pathogen found to be involved in abortion cases of humans, swine, and other mammals. Using broad-range DNA probes for bacterial RTX toxin genes, we cloned and subsequently sequenced a new operon named *paxCABD* encoding the RTX toxin PaxA in *P. aerogenes*. The *pax* operon is organized analogous to the classical RTX operons containing the activator gene *paxC* upstream of the structural toxin gene *paxA*, which is followed by the secretion protein genes *paxB* and *paxD*. The highest sequence similarity of *paxA* with known RTX toxin genes is found with *apxIIIA* (82%). PaxA is structurally similar to ApxIIIA and also shows functional analogy to ApxIIIA, since it shows cohemolytic activity with the sphingomyelinase of *Staphylococcus aureus*, known as the CAMP effect, but is devoid of direct hemolytic activity. In addition, it shows to some extent immunological cross-reactions with ApxIIIA. *P. aerogenes* isolated from various specimens showed that the *pax* operon was present in about one-third of the strains. All of the *pax*-positive strains were specifically related to swine abortion cases or septicemia of newborn piglets. These strains were also shown to produce the PaxA toxin as determined by the CAMP phenomenon, whereas none of the *pax*-negative strains did. This indicated that the PaxA toxin is involved in the pathogenic potential of *P. aerogenes*. The examined *P. aerogenes* isolates were phylogenetically analyzed by 16S rRNA gene (*rrs*) sequencing in order to confirm their species. Only a small heterogeneity (<0.5%) was observed between the *rrs* genes of the strains originating from geographically distant farms and isolated at different times.**

The gram-negative bacterium *Pasteurella aerogenes* was first isolated from porcine intestine and described as a gas-producing *Pasteurella*-like organism (30). Reported cases of isolation in animals have included the buccal flora of wild boars (33), the urine of rabbit, or the uterine cervix discharge of cow (3). In humans *P. aerogenes* has been isolated from lesions caused by cats, pigs, or wild boar (27, 30, 32).

Clinically, the isolation of *P. aerogenes* is mainly associated with abortion cases. The first case described in which *P. aerogenes* was directly involved as a pathogen was an abortion in swine, where it was isolated from several organs of the aborted fetuses (30). At least two additional cases of *P. aerogenes*-induced abortion in swine have been reported (13, 21). Abortion cases, where *P. aerogenes* could be responsible, were also reported in other mammals. It was isolated in pure culture from the uterus and peritoneal cavity of a rabbit which died 4 days after abortion (34). Also a human case is described where *P. aerogenes* could be isolated from a stillborn child and from its mother's vaginal vault (P. Thorsen, B. R. Moller, M. Arpi, A. Bremmelgaard, and W. Fredericksen, Letter, Lancet **343:** 485–486, 1994). During pregnancy, the mother had been working as an assistant on a pig farm. Other clinical cases are described in swine suffering from various diseases, where *P. aerogenes* was isolated from the lungs and respiratory system and quite often from intestines with gastroenteritis (3, 30), but its relevance as a primary pathogen in clinical findings other than abortion is doubtful. Despite the description of *P. aerogenes* as a potential pathogen, nothing is known about its possible virulence factors involved in pathogenicity.

RTX (repeats in the structural toxin) toxins, are a class of pore-forming protein toxins which are often found among various species of *Pasteurellaceae* and play an important role in pathogenicity (16). They were found in *Actinobacillus pleuropneumoniae* (ApxIA [20], ApxIIA [8], and ApxIIIA [7]), in *Actinobacillus actinomycetemcomitans* (AaltA [24]), *Pasteurella haemolytica* (LktA [28]), and *P. haemolytica*-like (PllktA [6]) and in *Actinobacillus suis* (AshA [5]). The operons are similarly organized in a *CABD* pattern where *C* codes for the activation protein, *A* encodes the structural toxin, and *B* and *D* code for proteins involved in the secretion of the toxin. We have therefore analyzed various strains of *P. aerogenes*, including strains from abortion cases in swine, for the presence of RTX genes by using a recently developed broad range detection system for this family of toxin genes (26). We describe a new RTX protein and its operon that was found in clinical *P. aerogenes* isolates and present a functional characterization of this toxin.

MATERIALS AND METHODS

Bacterial strains. A total of 13 *Pasteurella aerogenes* strains consisting of the type strain ATCC 27883^T and 12 field isolates were used in this study (Table 1). The field strains were freshly isolated at our diagnostic unit from clinical material of swine. *Actinobacillus pleuropneumoniae* serotype 2 reference strain ATCC 27089 (S1536) was included as control strain. For the analysis of the cohemolytic activity (CAMP) of RTX toxins (19) we used a beta-hemolytic *Staphylococcus aureus* expressing the sphingomyelinase and *Actinobacillus pleuropneumoniae* serotype 3 reference strain ATCC 27090 (S1421) secreting only ApxIIIA. Strains were grown either on Columbia Agar Base (Oxoid Unipath, Ltd., Basingstoke, Hampshire, England) or on 5% sheep blood agar plates at 37°C overnight.

Escherichia coli K-12 strains DH5α and HMS174 were used for gene cloning and expression, respectively. Strain JF522 harboring the *hlyBD* secretion genes on the plasmid pLG575 (29) was used in the CAMP test of recombinant *pax* constructs. All *Escherichia coli* strains were grown on Luria-Bertani broth supplemented, when necessary, with ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), or a combination of both for selection and maintenance of plasmids.

Probe preparation. Broad-range probes for RTX gene detection, leading to the discovery of a potential RTX gene in *P. aerogenes*, are described elsewhere (26). The *apxIIICA* and *apxIIIBD* probes from *A. pleuropneumoniae* were described previously (17). For generation of specific *P. aerogenes paxCA*, probe primers PAX14 (5′-ATTCGGGGATAACCATGCAC-3′; positions 306 to 325

* Corresponding author. Mailing address: Institute of Veterinary Bacteriology, University of Bern, Laenggass-Str. 122, CH-3012 Bern, Switzerland. Phone: 41-31-6312369. Fax: 41-31-6312634. E-mail: peter.kuhnert@vbi.unibe.ch.

TABLE 1. Swine isolates analyzed in this study

Strain no.	Isolation no.	RTX operon	CAMP	Origin	Pathological finding
P. aerogenes					
ATCC 27883T			−	Intestine	Diarrhea
JF2011	P811/97		−	Intestine	Diarrhea
JF1319	P1290/94	*pax*	+	Placenta	Abortus
JF2118	P325/98	*pax*	+	Placenta	Abortus
JF2006	P787/97	*pax*	+	Fetus	Abortus
JF2032	99/890	*pax*	+	Young piglet	Sepsis
JF2034	99/968		−	Intestine	Diarrhea
JF2039	P894/97		−	Liver	Sepsis
JF2142	P542/98		−	Intestine	Diarrhea
JF2072	P28/98		−	Liver	Sepsis
JF2101	99/1449		−	Intestine	Diarrhea
JF2185	P772/98		−	Bronchus	Pneumonia
JF2154	P577/98		−	Bronchus	Pneumonia
A. pleuropneumoniae					
ATCC 27089[a]		*apxII*, *apxIII*	+	Lung	Pleuropneumonia
ATCC 27090[b]		*apxII*, *apxIII*	+	Abscess	Periarticular abscess

[a] Serotype 2 reference strain S1536.
[b] Serotype 3 reference strain S1421.

on *paxC*) and PAX10 (5′-CGCACCACTTAATTCACGAG-3′; positions 2055 to 2035 on *paxA*) were used. For the *paxBD*-specific probe, primers PAX4 (5′-CT GGGATAAACAGCTAGCAAG-3′; positions 1077 to 1097 on *paxB*) and PAX15 (5′-TAACGTAAGCTGTTTGTCACG-3′; positions 925 to 945 on *paxD*) were used.

All probes were generated by PCR with digoxigenin-labeled dUTP (Roche Molecular Biochemicals, Rotkreuz, Switzerland). The labeling reaction was carried out in a 50-μl volume containing 5 μl of 10× PCR buffer, 20 pmol of primer (each), 1 mM deoxynucleoside triphosphate, 0.5 nmol of digoxigenin-11–dUTP, 2.5 U of *Taq* DNA polymerase (Roche Molecular Biochemicals), and 100 ng of genomic DNA. PCR conditions for the *pax*-specific probes were 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s.

DNA extraction and Southern blot. Extraction of genomic DNA was done by using either the QIAamp Tissue Kit (Qiagen, Basel, Switzerland) or the method of Pitcher et al. (31). Chromosomal DNA was digested by restriction enzyme, size separated on a 0.7% agarose gel, and vacuum transferred to a positively charged nylon membrane (Roche Molecular Biochemicals) by using an LKB 2016 VacuGene Vacuum Blotting Pump (Pharmacia LKB Biotechnology AB, Bromma, Sweden).

Hybridization with digoxigenin-labeled probes was done according to the manufacturer's instructions (Roche Molecular Biochemicals) in a rotating hybridization oven. Posthybridization washing steps were performed at middle stringency, defined as twice for 5 min at room temperature in 2× SSC–0.1 % sodium dodecyl sulfate (SDS) and twice for 15 min at room temperature in 0.2× SSC–0.1 % SDS (1× SSC is 0.15 M NaCl plus 0.01 M sodium citrate, pH 7.0). Chemiluminescent detection with CDP Star (Roche Molecular Biochemicals) as a substrate was done by using X-ray films.

Cloning and DNA sequence analysis. Chromosomal DNA of *P. aerogenes* JF1319 and plasmid pBluescriptII SK(−) were digested with corresponding restriction enzymes. Fragments were extracted from gel by using the Jetsorb Kit according to the manufacturer's instructions (Genomed, Bad Oeynhausen, Germany). After ligation, transformation of *E. coli* K-12 strain DH5α was done by the $CaCl_2$ procedure (2). Plasmid DNA was extracted by the alkaline lysis method (4), treated with RNase, and purified by phenol-chloroform extraction or by use of the Qiagen Miniprep Kit (Qiagen).

Sequential exonuclease III-generated deletions of cloned genes for subsequent DNA sequence analysis were carried out by using the double-stranded Nested Deletion Kit according to the manufacturer's protocol (Pharmacia Biotech, Dubendorf, Switzerland).

Sequencing was done by dye terminator-labeled fluorescent cycle sequencing by using Prism reagents and an ABI310 automated sequencer (PE Biosystems, Norwalk, Conn.). All sequences were edited on both strands by using the Sequencher program (GeneCodes, Ann Arbor, Mich.). Sequence comparisons were done by using BLAST (1).

Analysis of 16S rRNA genes. All strains investigated were tested genetically for their phylogenetic relationship by sequencing a 1.4-kb fragment of the 16S rRNA gene (*rrs*) as described previously (25). The *rrs* gene was amplified by using the universal 16S primers 16SUNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and 16SUNI-R (5′-GTGTGACGGGCGGTGTGTAC-3′). PCR was performed with a PE9600 automated thermal cycler with MicroAmp tubes (PE Biosystems) by using a polymerase with proofreading activity in order to avoid artifacts in the DNA sequences. The reaction was carried out in a 50-μl volume containing 5 μl of 10× PCR buffer, 20 pmol of primer (each), 1 mM deoxynucleoside triphosphate, 2.5 U of *Pwo* DNA polymerase (Roche Molecular Biochemicals), and 100 ng of genomic DNA as a template. PCR conditions were as follows: 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s. A final extension step for 7 min at 72°C was included. The PCR product was subsequently purified with the PCR Purification Kit (Qiagen) and sequenced as described above by using the set of primers described elsewhere (25).

Recombinant *pax* clones and CAMP test. Recombinant plasmids harboring either *paxCA* genes or the entire *paxCABD* operon were generated by PCR with the Expand Long Template PCR System (Roche Molecular Biochemicals) by using genomic DNA of JF1319 as a template. Plasmid pPaxCA was constructed by using primers paxCA-L (5′-GGACTAGTAGACATAAAAAAATACCAAT-3′; positions −95 to −76 on *paxC*) and paxCA-R (5′-CCGCTCGAGCATATT AGGATTGCTATTA-3′; positions 33 to 15 bp after the *paxA* stop codon), and plasmid pPaxCABD was constructed by using primers paxCA-L and paxBD-R (5′-CCGCTCGAGGTTTGATCTTCTACAAAT-3′; positions 21 to 4 bp after the *paxD* stop codon). After amplification the PCR products were digested with *Spe*I and *Xho*I and cloned into the corresponding sites of plasmid pBluescript II SK(−).

The CAMP test for cohemolytic activity (9) was performed on 5% sheep blood-agar plates by using a beta-hemolytic *Staphylococcus aureus* strain as described previously (19). The CAMP reaction was done with erythrocytes from different species. Blood was aseptically taken from swine, rabbits, horses, and humans in the presence of Alsever's solution. Agar plates were then prepared by overlaying Blood-Agar-Base (Oxoid, Hampshire, England) plates with Trypticase soy agar (BBL Becton Dickinson, Cockeysville, Md.) supplemented with 0.1% CaCl and 5% blood.

Expression of histidine-tailed fusion protein and mouse immunization. Primers PAXHIS-L (5′-GGACTAGTTGGTCTGCAATATGGGGTAAG-3′; positions 93 to 113 on *paxA*) and PAXHIS-R (5′-CGCGGATCCTTTTCCCTCT GGATCA-3′; positions 132 to 114 on *paxB*) were used to generate a PCR fragment from *paxA*. This fragment, containing the border between *paxA* and *paxB* genes, including the *paxA*-specific stop codon, was cloned in frame into the *Spe*I/*Bam*HI site of the pET-His vector. The recombinant histidine-tailed PaxA, missing the first 31 amino acids (aa) was expressed in *E. coli* HMS174. The culture was grown to an optical density at 650 nm of 0.5, induced with 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h, and then harvested. Cells were centrifuged 10 min at 2,500 rpm, and the pellet was dissolved in a 1/10 solution of sonication buffer (NaH_2PO_4 50 mM; NaCl, 300 mM) and sonicated in a Branson Sonifier 250 by using the microtip for 2 min, with an output control of 1 with cooling on ice. Sonicated cells were centrifuged for 20 min at 10,000 rpm, and the pellet was dissolved in 20 ml of 6 M guanidinium-HCl–0.1 M NaH_2PO_4–0.01 M Tris (pH 8.0) and incubated overnight with gentle shaking at 4°C. The dissolved sonication pellet was centrifuged 20 min at 10,000 rpm, and the supernatant was loaded onto a 2.5-ml Ni-nitriloacetic acid-agarose column (Qiagen, Hilden, Germany) which was prewashed with 10 ml of H_2O and 10 ml of 6 M guanidinium-HCl–0.2 M acetic acid and then equilibrated with 2 × 10 ml of 6 M guanidinium-HCl–0.1 M NaH_2PO_4–0.01 M Tris (pH 8.0). The loaded column was washed with 10 ml of 6 M guanidinium-HCl–0.1 M NaH_2PO_4–0.01 M Tris (pH 8.0) and then eluted with 10-ml aliquots of elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris) at pH values of 8.0, 7.0, 6.0, 5.0, and 4.5. Fractions of 1 ml were collected and analyzed on an SDS-gel. Fractions containing the

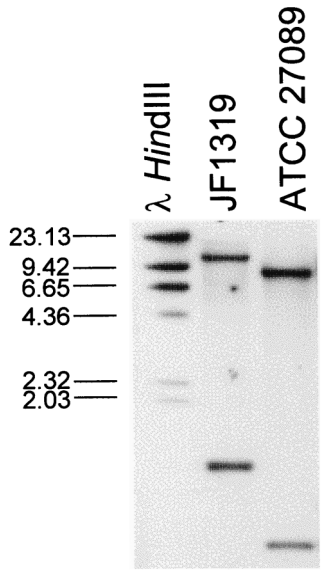

FIG. 1. Southern blot of *P. aerogenes* and *A. pleuropneumoniae* with *apxIIICA* as probe. Genomic DNA of *P. aerogenes* JF1319 from a swine abortion case and *A. pleuropneumoniae* serotype 2 reference strain ATCC 27089 (S1536) was digested with *Eco*RI. After electrophoresis on a 1% agarose gel and transfer to nylon membrane, the filter was hybridized with the digoxigenin-labeled *apxIIICA*-derived probe.

histidine-tailed PaxA were pooled, dialyzed against TE (0.1 M Tris, 1 mM EDTA; pH 7.5), and used for mouse immunization.

Nucleotide accession numbers. The 16S rRNA gene sequences were deposited under GenBank accession numbers U66491 (ATCC 27883^T), U66492 (JF1319), AF139577 (JF2011), AF139578 (JF2118), AF139579 (JF2006), AF139580 (JF2032), AF139581 (JF2034), AF139582 (JF2039), AF139583 (JF2142), AF139584 (JF2072), AF139585 (JF2101), AF139586 (JF2185), and AF139587 (JF2154). The sequence of the *pax* operon is deposited under accession number U66588.

RESULTS

Cloning and sequencing the *pax* operon. Based on the observation that a *P. aerogenes* field isolate (JF1319) hybridized with a set of broad-range DNA probes for the detection of RTX toxin genes (26), we characterized the hybridization signal in more detail. The strongest hybridization was seen with a subset consisting of the *apxIIIA*-derived gene probe. Genomic DNA of *P. aerogenes* JF1319 and of the *A. pleuropneumoniae* serotype 2 reference strain ATCC 27089 (S1536), used as a control for the *apxIII* operon, was digested with *Eco*RI and blotted onto nylon membranes. Southern blots were subsequently hybridized with a probe specific for *apxIIICA* (Fig. 1). In the control strain *A. pleuropneumoniae* ATCC 27089 this results in the detection of two bands as expected from the sequence of the *apxIIIA* operon. One band at 730 bp resulted from a fragment containing the 3′ half of *apxIIIC* and a short part of *apxIIIA*. The other band at 9 kb covers the 3′ part of *apxIIIA* as well as the two genes *apxIIIB* and *apxIIID*, coding for the secretion proteins. In *P. aerogenes* JF1319, two bands were observed which clearly differed from *apxIII*. The bands were located at 1.3 and ca. 11 kb, suggesting a different gene (Fig. 1). When an *apxIIIBD*-specific probe was used, the 11-kb band of *P. aerogenes* also hybridized analogous to the 9-kb band of *A. pleuropneumoniae* (data not shown). This shows that *P. aerogenes* also contains RTX secretion genes *B* and *D* and suggests the presence of a complete RTX operon.

The genes of the putative RTX determinant of *P. aerogenes* field isolate JF1319 were cloned and sequenced. For this purpose a 3.3-kb *Bgl*II fragment hybridizing to the *apxIIICA* probe was cloned into the *Bam*HI restriction site of plasmid pBluescriptII SK(−) resulting in pJFFPAE1. This clone was used as a probe to find additional fragments covering the RTX operon. Thereby a 2.8-kb *Pst*I fragment and a 5.5-kb *Hin*dIII fragment were cloned, resulting in pJFFPAE2 and pJFFPAE5, respectively. By using subclones of these basic clones and by using in addition the primer walking method we were able to sequence the complete operon in both directions. The new RTX operon was named *pax* (for *P. aerogenes* RTX toxin) in accordance with nomenclature of RTX toxins (18). Accordingly, the gene encoding the potential structural RTX toxin *paxA*, the gene for the activator *paxC*, and the two genes coding for putative secretion genes *paxB* and *paxD*. A map of the *pax* operon, which shows the characteristic features of RTX operons, and the basic clones used for its sequence determination are shown in Fig. 2.

Characterization of the *pax* operon. The *paxC* gene is 510 bp long coding for a putative 169-aa (17.5-kDa) activator protein. It shows 82% similarity to the *apxIIIC* gene. The *paxA* gene is 3.15 kb long, encodes a presumed 1,049-aa toxin of 107.5 kDa and also shows 82% similarity to *apxIIIA*. The deduced protein sequence of PaxA (Fig. 3) contains seven characteristic glycine-rich nonapeptide repeats based on the consensus L/I/F-X-G-G-X-G-N/D-D-X (36). Four similar repeats precede these classical patterns. The potential secretion protein genes *paxB*, which spans 2,136 bp and codes for a calculated 711-aa protein of 73 kDa, and *paxD*, which encodes a presumed 477-aa protein (49 kDa), are also present on the operon and show typical features of ABC transporters. The *paxB* gene is 83% similar to *apxIIIB*, the *paxD* gene 82% similar to *apxIIID*. Table 2 summarizes the similarities of the *pax* DNA and amino acid

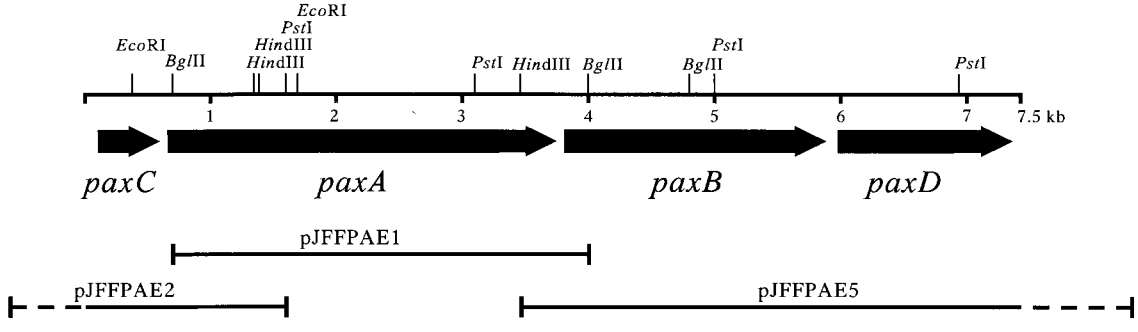

FIG. 2. Restriction map of *pax* operon from *P. aerogenes* JF 1319 and positions of the different clones used for determination of its sequence. The black arrows represent the four genes in the *pax* operon, with the arrowheads showing the direction of transcription.

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   1  MSTWSSMLADLRKQAEIAKQQAKKGIDVTKNGLQYGVSQVKLQALAAGKS
  51  IQKYGNKLVLVIPKDYDVNTGNGFFDLAKAAEELGIQVKYIDRNDLEIAH
 101  KSLGVTDQFLGLTERGLTLFAPQLDKFLQQHSKISNVVGSSTGDTVNKLA
 151  KSQAIISGVQSVLGSVLAGINLNEAIISGGSELELAKAGVDLASELVGNI
 201  AKGTATIEAFSEQIQNFGKLVQNAKGLGGVGQQLQHISGSALSKTGLGLD
 251  IISSLLSGVTASFTLADKNASTSTKVAAGFELSNQVIGGITKAVSSYILA
 301  QRLAAGLSTTGPAAALIASSISLAISPLSFLRVADNFNRSKDIREFAERF
 351  KKLGYEGDKLLSDFYHEAGTIDASITTISTALSAIAAGTAAASAGALVGA
 401  PITLLVTGITGLISGILEFSKQPMLEHVASKLGTKIEEWERKYGKNYFEN
 451  GYDARHKAFLEDSLSLLSSFNKQYETERAVLITQQRWDEYIGELAGVTGK
 501  GDKISSGKAYVDYFEEGKLLAKKPDDFNRVILDPKKGKIDISNSQTSTLL
 551  KFVTPLLTPGTESRKRTQTGKYEYVTKLDVNGINQWEVNGVKEKGAVYDF
 601  TNLIQHVHISSSVARGEEYREVRLVSRLGKGNDKVFLASGSAEIHAGDGH
 651  DVVYYDKTDTGLLMVDGTQATKQGDYTVTRELSGATQILREVVKNQKSSV
 701  GSRQETVEYRDNELAQSGNSNLKAKDNLYSVEEIIGSNHRDEFKGSKFRD
 751  IFHGADGDDLLNGNDGDDILYGDKGNDELRGDNGNDQLYGGEGNDKLFGG
 801  NGNNYLSGGDGDDELQVLGNGFNVLRGGKGNDKLYGGAGSDFLDGGEGDD
 851  YLAGGEGNDFYVYRSTSGNHTIYDQGKSSDSDTLYLSDLTFDRLLVEKVD
 901  NNLVFKPSDHNSNRGSLTIKDWFKTGHGYNHKLEQIVDKNGRKLTSDNLE
 951  THFNGTPKTNLLGYTAENQNESNLSSLKTELGKIISSAGNFGLAKQGNNN
1001  HSAALNNDVDKLISSASSFATAQMGGSGIGLLPSNNANSTILSGLARTA
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FIG. 3. Amino acid sequence of PaxA. The seven consensus glycine-rich nonapeptide sequences are double underlined. The four preceding similar nonapeptide repeats are underlined.

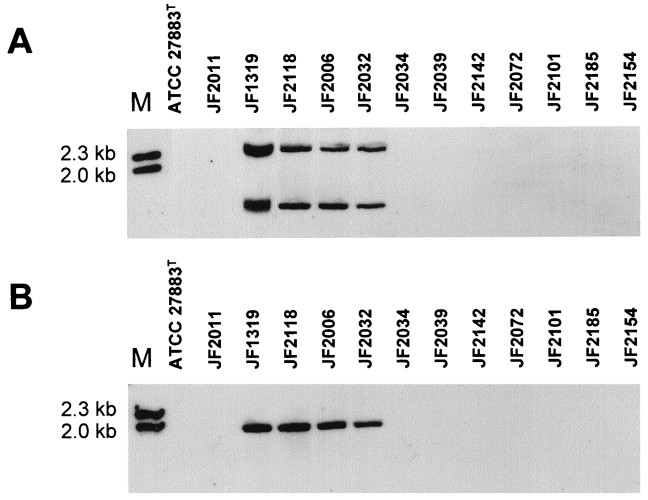

FIG. 4. Southern blot of *P. aerogenes* strains. Genomic DNA of the type strain ATCC 27883^T and 12 clinical isolates was digested with *Pst*I, electrophoresed on 1% agarose gel, and transferred to nylon membranes. (A) Hybridization with digoxigenin-labeled *paxCA* derived probe. (B) Hybridization with digoxigenin-labeled *paxBD*-derived probe. M, λ*Hin*dIII marker, showing the 2.3- and the 2.0-kb fragments.

sequences with RTX genes described in other *Pasteurellaceae* and the alpha-hemolysin of *E. coli*.

Presence of *pax* in *P. aerogenes* strains. In order to determine the prevalence of *pax* in this species, all *P. aerogenes* strains (Table 1) were screened for *pax* by Southern blots with *Pst*I-digested genomic DNA and probes specific for *paxCA* and *paxBD*. The results in Fig. 4 show the characteristic bands for the *pax* operon in four *P. aerogenes* strains isolated from abortus cases or from a young piglet with septicemia originating from geographically distant farms and isolated in different years (JF1319, JF2118, JF2006, and JF2032). The *P. aerogenes* type strain, as well as the resting eight strains that were isolated from clinical material of pathologies other than abortus, did not show any signal with the *pax*-derived gene probes (Table 1).

Identification of *P. aerogenes* by sequence analysis of the 16S rRNA gene (*rrs*). Since the phenotypic identification of *P. aerogenes* has shown to be sometimes ambiguous, we further identified all strains selected for this study by sequencing of the 16S rRNA gene (*rrs*). A few strains which were initially identified by phenotypic methods to be *P. aerogenes* were revealed by the sequence data to be other species of the family of *Pasteurellaceae* and were thus not included in this study. Among the *P. aerogenes* strains isolated from clinical material, variations in the *rrs* sequences ranged from two to six different nucleotides (<0.5% variation) compared to the type strain *rrs* sequence. Some strains showed ambiguous bases at a few positions, indicating the presence of more than one *rrs* operon. Strains JF2011, JF2154, and JF2039, as well as strains JF1319 and JF2118, had identical sequences. By comparison of our sequence of the type strain with the one previously deposited by others (accession number M75048), we could resolve all of the unidentified bases in the latter. In addition, we detected three differences between sequence M75048 and our sequence of the type strain (U66491).

TABLE 2. Similarity of the nucleotide and amino acid sequence of Pax proteins and their genes to other RTX determinants in *Pasteurellaceae* and to Hly of *E. coli*

Locus	DNA or protein	% Identity (DNA) or % similarity (protein)			
		PaxC	PaxA	PaxB	PaxD
ApxI	DNA	63	63	74	70
	Protein	72	76	94	80
ApxII	DNA	67	62		
	Protein	78	80		
ApxIII	DNA	82	82	83	83
	Protein	91	94	97	95
Lkt	DNA	63	65	74	68
	Protein	76	82	93	81
Plkt	DNA	63	65	74	67
	Protein	81	82	93	82
Aalt	DNA	68	60	72	64
	Protein	74	73	91	78
Hly	DNA	66	62	71	61
	Protein	74	78	94	78

Functional analysis of the PaxA toxin. Since *paxA*-containing *P. aerogenes* did not show direct hemolytic activity on sheep and swine erythrocytes, we performed the CAMP test (9) for cohemolytic activity of this new RTX protein. Cohemolytic activity is known to be associated with Apx toxins, including ApxIIIA (19, 22). For this purpose all *P. aerogenes* strains were grown in the vicinity of a beta-hemolytic *S. aureus*. As a CAMP-positive control for RTX toxins we used the *A. pleuropneumoniae* serotype 3 reference strain. This strain only secretes the nonhemolytic, but CAMP-positive, cohemolytic ApxIIIA which shows hemolysis only in the diffusion zone of the shingomyelinase of *S. aureus*. Whereas all four *pax*-positive isolates produced a clear hemolytic zone comparable to the ApxIIIA control, none of the *pax*-negative strains showed a CAMP effect (Fig. 5 and Table 1).

In order to prove that the cohemolytic activity is due to PaxA, we constructed two recombinant plasmids containing either the entire *pax* operon (plasmid pPaxCABD) or only the *CA* genes (plasmid pPaxCA). *E. coli* DH5α was then transformed with these plasmids. In addition, strain 5K containing *hlyBD* genes on a plasmid (29) was transformed with plasmid pPaxCA. The results of using the various clones in the CAMP test are shown in Fig. 5. DH5α becomes CAMP positive when containing the entire *pax* operon (Fig. 5, lane 9). No CAMP effect is seen with the wild-type *E. coli* host strain (Fig. 5, lane 7) or the strain containing the vector with the *CA* genes (Fig. 5, lane 8). On the other hand, strain 5K with *hlyBD* alone shows no hemolysis (Fig. 5, lane 10) but it becomes cohemo-

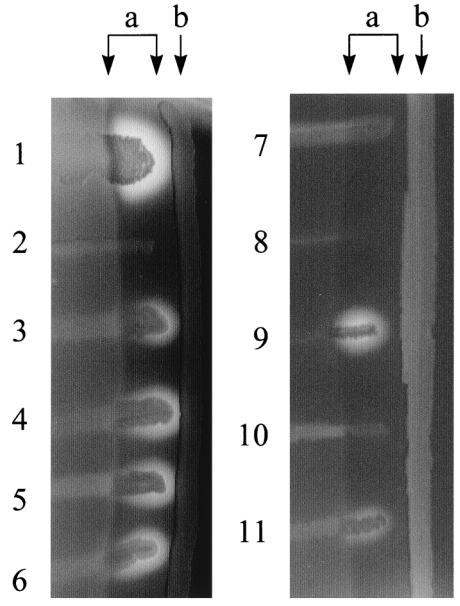

FIG. 5. CAMP test with *P. aerogenes* isolates and recombinant *E. coli* K-12 strains. Strains were grown in the vicinity of *S. aureus* (b). The diffusion zone of the sphingomyelinase is also indicated (a). The four *pax*-positive *P. aerogenes* isolates JF1319 (lane 3), JF2006 (lane 4), JF2032 (lane 5), and JF2118 (lane 6) show a distinct zone with complete hemolysis, as does *A. pleuropneumoniae* serotype 3 reference strain ATCC 27090 (lane 1). The *P. aerogenes* type strain (lane 2), which does not contain the *pax* operon, shows no CAMP effect. The CAMP effect in *P. aerogenes* is due to the presence of *pax*, as shown with an *E. coli* K-12 DH5α transformed with the plasmid pPaxCABD containing the complete functional operon (lane 9). Neither the DH5α wild-type strain (lane 7) nor the strain containing only *paxCA* genes on plasmid pPaxCA (lane 8) show the CAMP reaction. A mild CAMP effect is also seen in *E. coli* 5K strain harboring the *hlyBD* genes on plasmid pLG575 and plasmid pPaxCA (lane 11) but not in the control strain containing only *hlyBD* (lane 10).

lytic if *paxCA* genes are also present (Fig. 5, lane 11). The less intensive cohemolysis of this last construct might be due to the fact that the *hlyBD* gene products secrete PaxA less efficiently than does the endogenous *paxBD*-encoded secretion machinery.

In order to determine whether the cohemolytic CAMP effect was specific to a given host, blood-agar plates prepared with erythrocytes from different species were used. For both RTX toxins, PaxA and ApxIIIA, the cohemolytic zone differed on the various erythrocytes (Table 3). Whereas on sheep blood the cohemolysis was generally more intense, it was less strong on human and rabbit erythrocytes and weak on pig and horse erythrocytes. On pig erythrocytes both PaxA and ApxIIIA showed about the same strength of cohemolysis, while on erythrocytes of the other species PaxA was less active than ApxIIIA.

Purified recombinant polyhistidine-tailed PaxA protein showed a serological cross-reaction with the ApxIIIA toxin from *A. pleuropneumoniae*, thus giving further evidence of the close relationship between these two toxins (data not shown).

DISCUSSION

P. aerogenes is known to belong to the normal intestinal flora of swine, as well as to act as an opportunistic pathogen (21, 30). Human cases of infection are rare and may occur after being bitten or gored by swine or via dog and cat bites or scratches. Due to its dual role as normal flora and an opportunistic pathogen, its pathogenicity is poorly understood and difficult to investigate. One study was done by inoculating mice with different strains of *P. aerogenes*. The various strains affected the mice heterogeneously, and two of the ten strains tested led to death after 24 h (33).

Despite its recognition as a mainly opportunistic pathogen, there are sporadic reports of *P. aerogenes* as a pathogen in abortion cases. Already in the first description of *P. aerogenes*, McAllister and Carter (30) describe an abortion case as the only clinical finding where *P. aerogenes* was involved as a primary pathogen. Other cases of abortion in swine were later reported by Hommez and Devriese (21), as well as by Fodor et al. (13). Thorsen et al. (Letter, Lancet **343:**485–486, 1994) recently published a case report of human abortion due to *P. aerogenes*.

We report here the identification of a new RTX toxin gene, *paxA*, and its corresponding cohemolytic phenotype, which associates with specific *P. aerogenes* strains isolated from abortion cases in swine or from septicemia of young piglets. Other *P. aerogenes* strains which were isolated from different clinical samples of pigs with uneven pathological findings were devoid of the *pax* operon and did not produce CAMP cohemolysis.

The *pax* operon shows high similarity to the *apxIII* operon of *A. pleuropneumoniae*. Due to its high similarity (94%) to ApxIIIA and due to its immunological relatedness to ApxIIIA the activity of PaxA could be similar. ApxIIIA is nonhemolytic but strongly cytotoxic for alveolar macrophages and neutrophils (16) and shows a cohemolysis with the *S. aureus* sphingomyelinase known as the CAMP reaction (19). The same cohemolytic effect was observed with *P. aerogenes* harboring the *pax* operon, whereas none of the *pax*-negative isolates showed the CAMP effect. The cohemolytic CAMP reaction of PaxA was observed on erythrocytes from different hosts. This finding is in agreement with other hemolytic or cohemolytic RTX toxins for which also no host specificity as determined by erythrocyte lysis was found.

We could demonstrate that the cohemolytic activity in *P. aerogenes* is specifically caused by the presence of the *pax* operon (Fig. 5). Transforming *E. coli* K-12 with the entire *pax* operon was sufficient to convert this CAMP-negative strain into a CAMP-positive one. The same phenotype conversion was observed when transforming only the *paxCA* genes into a K-12 strain containing functional genes for the *hly*-specific secretion proteins (*hlyBD*) but not when the *paxCA* genes alone were present. This shows that PaxA must be secreted via a type I secretion system to exert its activity. This set of experiments shows further that the PaxA protein can be secreted not only by its own *paxBD*-encoded secretion system but also by the secretion system encoded by the *hlyBD* genes of the *E. coli* alpha-hemolysin operon. Nevertheless, the CAMP effect in the latter (Fig. 5, lane 11) seems weaker than in the strain harboring the entire *pax* operon. This could be the result of a less-efficient secretion of *paxA* via the *hly* secretion pathway compared to its own specific pathway.

TABLE 3. CAMP cohemolytic activity of PaxA and ApxIIIA on erythrocytes of different species

Blood source	Reaction[a] with:		
	Pax$^+$ *P. aerogenes*	Pax$^-$ *P. aerogenes*	ApxIII$^+$ *A. pleuropneumoniae*
Human	+	−	++
Horse	−	−	(+)
Pig	(+)	−	(+)
Rabbit	+	−	++
Sheep	++	−	+++

[a] +++, strong CAMP reaction; (+), distinct CAMP cohemolysis still visible.

Interestingly, no consensus sequence for a ribosomal binding site can be found in front of any of the four genes of the *pax* operon. However, the cloned *paxCABD* operon, including a region of approximately 100 bp upstream the first gene *paxC*, was well expressed in the functional protein in *E. coli* hosts, resulting in the characteristic CAMP effect.

The CAMP cohemolytic activity has also been described for other *Pasteurellaceae* which are known pathogens as *Pasteurella haemolytica* (15) (now *Mannheimia haemolytica*), *Actinobacillus pleuropneumoniae* (23), or *Pasteurella granulomatis* (35) (now *Mannheimia granulomatis*). The precise role of the cohemolytic activity of the PaxA toxin has still to be established. However, the CAMP effect serves as a useful test that will allow investigators to rapidly differentiate PaxA-toxigenic strains from other *P. aerogenes* and to study the role of PaxA-producing *P. aerogenes* in abortion in swine.

A detailed genotypic study of the *rrs* sequence of all strains allowed us to confirm solidly the species *P. aerogenes* for all strains used in this study. A few strains which were initially identified phenotypically as *P. aerogenes* were revealed to be other *Pasteurellaceae* after 16S rRNA gene sequence analysis and were thus excluded. This revealed the importance of genotypic verification of the species *P. aerogenes*, since unambiguous identification of the species *P. aerogenes* by phenotypic means seems to be hampered by certain biochemical reactions. Comparison of the 16S rRNA (*rrs*) gene sequences of the 13 *P. aerogenes* strains included in this study revealed only minor variation in its *rrs* genes, i.e., $<0.5\%$. This is within the range of intraspecies variation (10, 14). Based on their *rrs* sequence, all strains map at the very same position on the phylogenetic tree of *Pasteurellaceae* described by Dewhirst et al. (12). There was no correlation between the presence of the *pax* operon and the 16S rRNA gene sequences, which raises the hypothesis that *pax* might not be clonal and therefore could be located on a relatively mobile DNA. It could thereby be lost or acquired by certain *P. aerogenes* strains. This would help to explain the ambiguous role of *P. aerogenes* as a pathogen leading to severe complications such as abortus or septicemia of newborn piglets in certain cases due to PaxA-toxigenic strains and as a bacterium of low epidemiologic impact in many other circumstances (nontoxigenic strains). In this respect the detection of *pax* could be an indicator for virulent representatives of this species. The role of PaxA in abortion remains speculative for the moment. Nevertheless, since RTX toxins are known inducers of cytokines such as interleukin-1 and tumor necrosis factor, they are thought to have an immunomodulating effect (11). Therefore, it is conceivable that in the special immune status of pregnancy this modulating effect could finally lead to abortion.

In summary, the new RTX toxin PaxA is the first potential virulence factor described in *P. aerogenes*. PaxA showed cohemolytic activity in the CAMP test. This simple diagnostic test allows researchers to differentiate PaxA-toxigenic *P. aerogenes* from other, probably less virulent *P. aerogenes* strains. Since PaxA and its operon *paxCABD* was specifically found in *P. aerogenes* isolated from cases of abortion or septicemia in newborn piglets, we speculate that PaxA is involved in the virulence of *P. aerogenes*.

ACKNOWLEDGMENTS

We thank Y. Schlatter and M. Krawinkler for technical assistance.

This work was supported by grant 5002-38920 from the Swiss National Science Foundation.

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Editor: J. T. Barbieri